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Application of high-performance liquid chromatography coupled to nuclear magnetic resonance spectrometry, mass spectrometry and bioassay for the determination of active saponins from *Bacopa monniera* Wettst.

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Abstract

Reversed-phase high-performance liquid chromatographic separation coupled to (structurally informative) spectroscopic methods like NMR and MS and an efficient bioassay have been used to determine the active compounds from a crude fraction of *Bacopa monniera*. The fraction containing a mixture of saponins with closely related structures was found to show a significant anthelmintic activity against *Caenorhabditis elegans* (used as a model test organism for determining anthelmintic activity). The activity was correlated to two dammarane type triterpenoidal saponins containing at least three sugar units. The optimization of separation for 1 mg of the crude sample on column and the sensitivity of on-flow one- and two-dimensional NMR experiments to the high-molecular-mass compounds (M_r 890–930) has been demonstrated. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Bacopa monniera*; Saponins; Bacosides; Bacopasaponins

1. Introduction

The potential of liquid chromatography–nuclear magnetic resonance (LC–NMR) and LC–mass spectrometry (MS) for a rapid and direct screening of crude plant extracts for useful products has recently gained considerable attention [1–4]. Hence, this enables the identification of secondary metabolites at a very primary stage avoiding the time consuming isolation of pure components. Further using LC–coupled bioassay, the biological activity could be directly correlated to the identified compounds [5]. These hyphenated techniques in combination lead to a rapid and efficient analysis of plants for compounds of phytochemical or pharmacological interest.

The case in study is *Bacopa monniera* W. (Scrophulariaceae), used in traditional medicine as a reputed nervine tonic, cardiotonic and diuretic [6]. The plant is well studied chemically and much of the activity is attributed to the dammarane type triterpenoidal saponins [7]. The saponins are known to be present as a complex mixture of closely related structures namely bacopasaponins A–D, bacosides A_1 and A_3. Hence, posing a challenge to optimize the separation conditions for on-line screening [8–11].

Here, we report the applications of the reversed-phase high-performance liquid chromatographic separation coupled with structurally informative spectroscopic methods, NMR and MS to an active

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fraction of *B. monniera*. The in vitro bioassay with *Caenorhabditis elegans* as a test organism is well known for the off-line screening of anthelmintic activity. The recent demonstration of the high-performance liquid chromatography (HPLC)–coupled version of the assay was applied to correlate the activity to the identified compounds [5]. The strategy led to the straightforward identification of the saponins which were structurally closely related, as active compounds. The saponins differed only in the nature of the sugar units in the glycosidic chain and the position of the olefinic side chain in the aglycone. The comparison of the spectroscopic data with the data from literature and authentic samples confirmed the sensitivity of the on-line and off-line HPLC–NMR technique for such high-molecular-mass compounds.

2. Experimental

2.1. Extraction and bioassay-guided fractionation

The bioassay for anthelmintic activity with *C. elegans* as test organism and the preparation of cell cultures was done according to Simkin and Coles [12]. The test medium was made by adding 10 ml of *E. Coli* to 100 ml M9 buffer plus 5 mg ampicillin and 1000 U nystatin obtained from Sigma (Deisenhofen, Germany). Two ml of the test medium was placed in each well of a tissue culture plate (Renner). One mg of the sample was dissolved in 50 μl dimethyl sulfoxide (DMSO) and 10 μl of this solution was added in each well together with 10 μl of a suspension of *C. elegans* containing 20–30 worms of mixed age. After incubation at 20°C for seven days, the plates were observed under a binocular microscope (obtained from Nikon) and assessed for increase in number and movement. The extracts were processed guided by the bioassay to obtain the active fraction.

The plant material *B. monniera* Wettst. (Scrophulariaceae) was obtained from the local market in Bangalore, India. The dried plant material was defatted in light petroleum (30–50°C) for 48 h and was further subjected to succesive extractions in solvents of increased polarity like ethyl acetate, methanol and 50% aq. methanol. The viscous mass obtained from the methanolic extract was partitioned between *n*-butanol and water, the butanol layer was concentrated to give a residue weighing 5.93 g. The residue was dissolved in 90% aq. methanol and filtered through a bed of RP C_{18} material. The filtrate was evaporated to give 5.41 g of the crude extract free of chlorophyll. The crude extract on gel permeation chromatography using Sephadex LH-20 and methanol as the eluent gave seven fractions (F1–F7).

2.2. HPLC–bioassay

For the HPLC–bioassay evaluation, F1 was injected at a concentration of 0.4 mg in 50 μl acetonitrile (MeCN) onto an analytical HPLC system composed of a Bischoff HPLC Compact 1709 pump, a Bischoff HPLC central processor and a Bischoff LC gradient mixer, obtained from Bischoff Chromatography (Leonberg, Germany) attached to a MD-910 multiwavelength detector from Jasco (Tokyo, Japan). The separation was performed using a LiChrospher 125×5 mm, 5 μm RP C_{18} HPLC column from E. Merck (Darmstadt, Germany) and a gradient composed of MeCN (Riedel-de Haen, Seelze, Germany) and water. The solvent program used was an isocratic run with 30% aq. MeCN for 20 min followed by an increase to 80% aq. MeCN at a flow-rate of 0.8 ml/min to elute the strongly retained compounds. The eluent of the column was collected in the test wells of the tissue culture plate (24 wells with a volume of 2 ml) at a rate of 1 min/well. The solvent in the wells was evaporated under vacuum in a desiccator. This was followed by the bioassay as described above.

2.3. HPLC–MS

For HPLC–MS, the separation was performed with a Model ABI 140B pump and a Model ABI detector 785A, obtained from Applied Biosystem Instruments using similar column and chromatographic conditions as described above. The chromatographic system was coupled to a Finnigan MAT TSQ 700 MS atmospheric pressure chemical ionization (APCI) interface (Finnigan, San Jose, CA, USA). The spectra were recorded in the positive ion mode.

2.4. HPLC–NMR

Similar chromatographic conditions as given above were applied to the HPLC–NMR analysis (^2H_2O-acetonitrile). For the HPLC–NMR run under ^2H_2O-methanol, a gradient from 60% to 90% aq. methanol over 30 min was used. The HPLC–NMR system consisted of a Varian 9012 pump and a Varian 9050 UV detector connected to a Varian Unity INOVA (500 MHz) spectrometer (Palo Alto, CA, USA) equipped with a pulsed field gradient (PFG)-LC–NMR flow probe with an active cell volume of 60 μl. Twenty mg of extract was diluted in 1 ml of methanol and 50 μl of this solution was used for the online-detection of one- (1D) and two-dimensional (2D) NMR-resonances. NMR experiments were carried out with solvent supression technique WET [13].

3. Results and discussions

A systematic bioassay-guided fractionation for anthelmintic activity (against *C. elegans*) was adopted to screen the extracts of *B. monniera*. The experiments clearly indicated the methanolic extract to be active at a concentration of 100 ppm. The extract was filtered through a bed of RP C_{18} and eluted with 90% aq. methanol, by which the chlorophyll in the extract was efficiently separated. The gel permeation chromatography of the residue (free of chlorophyll) using Sephadex LH-20 and methanol as eluent offered seven fractions (F1–F7), out of which F1 (containing compounds of higher molecular mass) showed significant anthelmintic activity. In order to identify the active principle rapidly, further biological and spectroscopic analysis of the crude fraction F1 was done exclusively by HPLC coupled techniques.

As separation plays an important role for on-line coupled analysis, the HPLC conditions were optimized such that a baseline-to-baseline separation was obtained for the compounds of F1. The LiChrospher RP-18 HPLC analytical column using 30% aq. MeCN at a rate of 0.8 ml/min was found to be ideal with good separation upto 1 mg of the F1 loaded on column (Fig. 1). The separation factor (α) for the

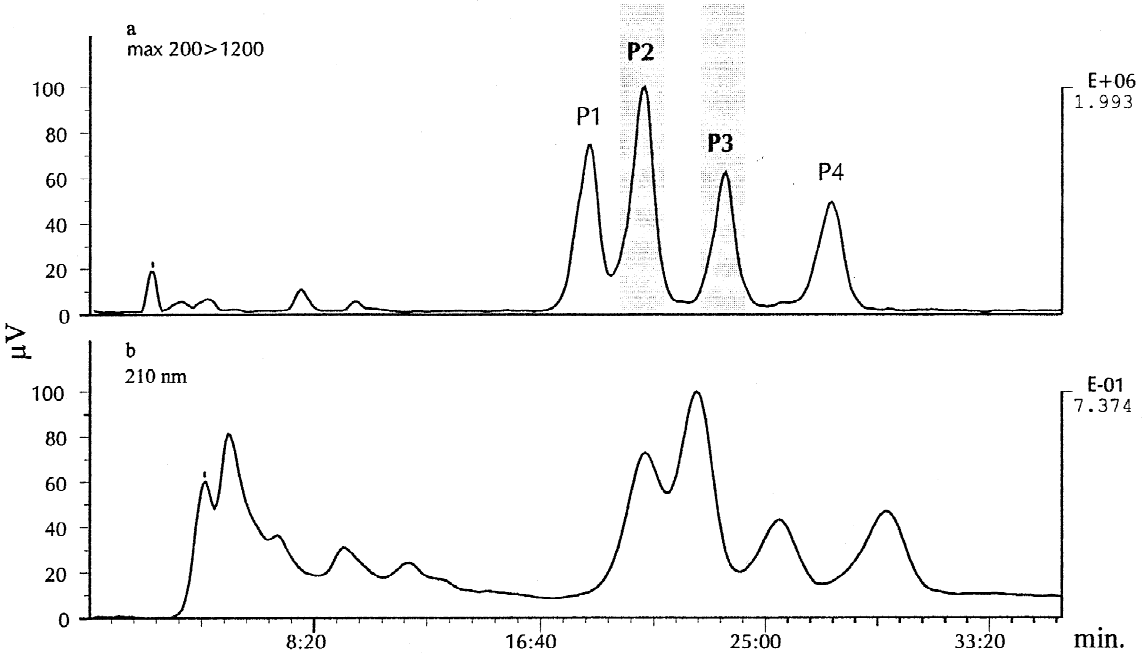

Fig. 1. (a) Relative ion chromatogram of F1 indicating the active peaks. (b) UV trace. See text for chromatographic conditions.

major peaks seen in the chromatogram was determined to be between 1.13 and 1.18. The separation conditions were also found suitable for the bioassay to concentrate the peaks in each well without any mixing that may lead to misinterpretation. Hence, the injection concentration chosen was 0.4 mg/50 μl with respect to the minimum concentration found to be active in the off-line assay and the eluent was collected at a rate of 1 min per well. The test wells containing the active fraction corresponded to peaks 2 (P2) and 3 (P3) in the chromatogram.

The HPLC–MS run was obtained using both electrospray ionization (ESI) and APCI interfaces in the positive and negative ion modes. The MS spectra obtained using APCI interface in the positive ion mode was used to analyse the data. The four major peaks of the active fraction identified by the retention time and peak pattern gave a molecular ion peak $[M+H]^+$ at m/z 929 for peaks 1 (P1) and 2 (P2).

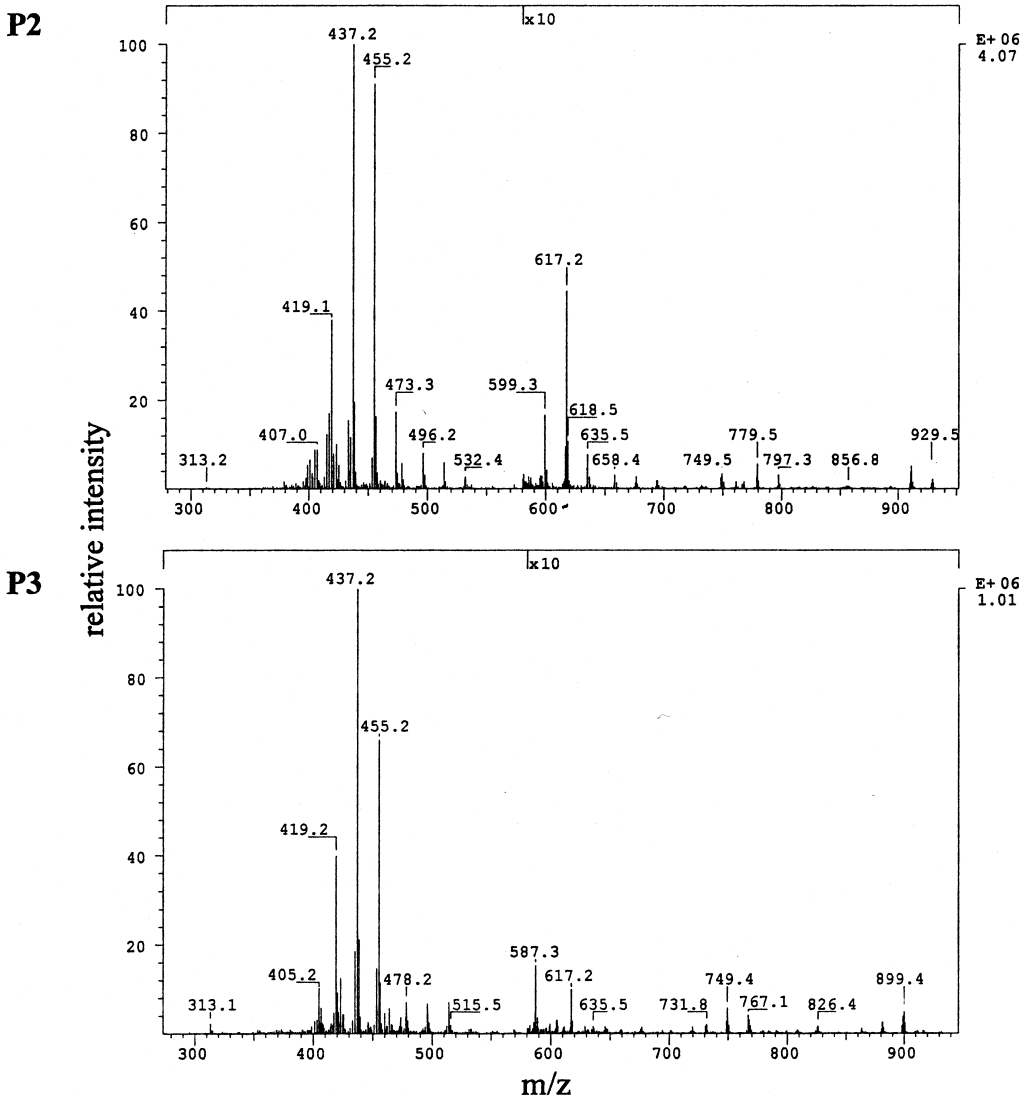

Fig. 2. HPLC–APCI-MS spectra of the active peaks P2 and P3 indicating the characteristic signals.

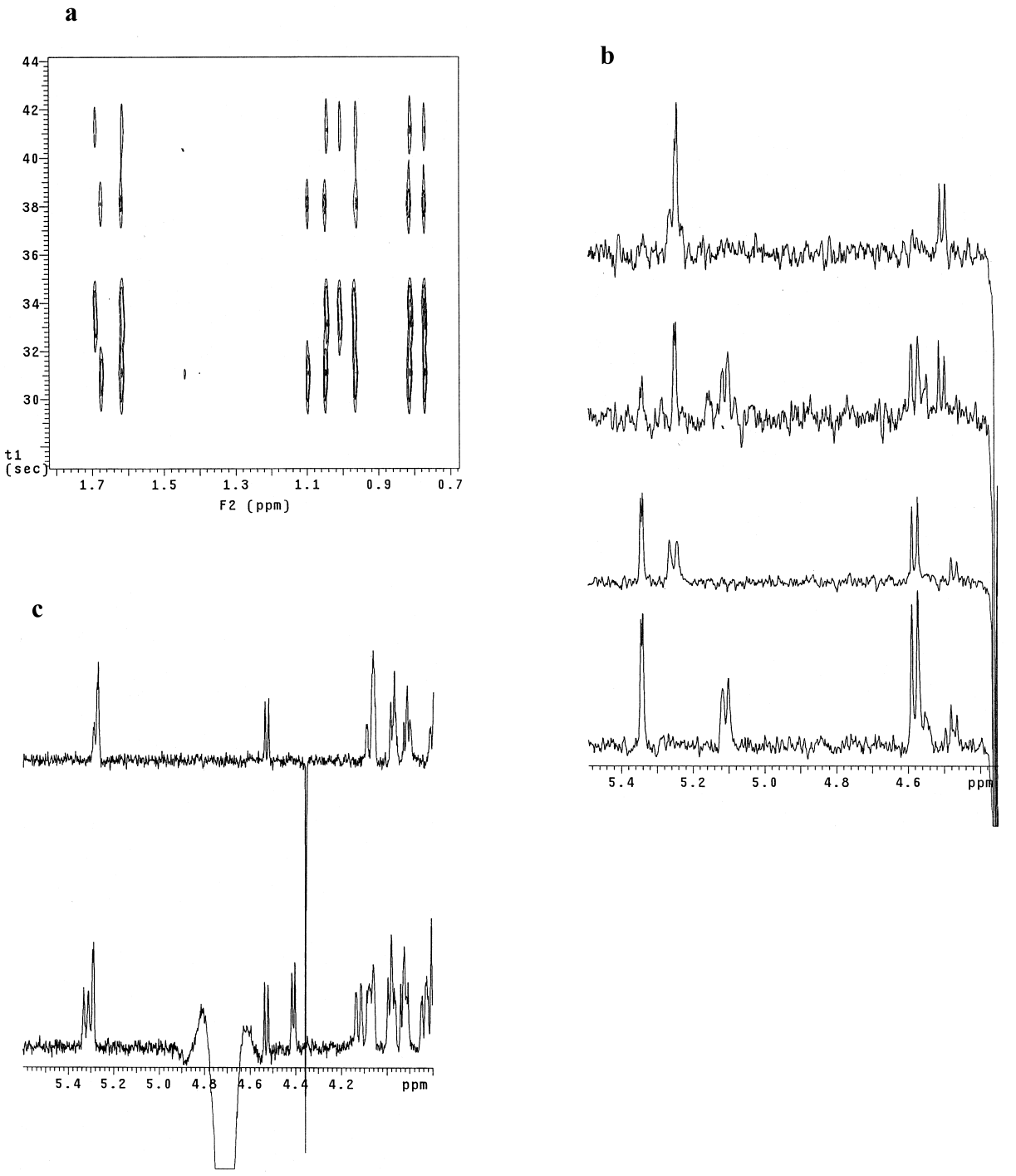

Fig. 3. (a) On-flow LC–NMR spectra indicating the ^{1}H-NMR resonances in the methyl region of the major peaks from F1 under MeCN–$^{2}H_2O$ conditions. (Plotted with same expansion). (b) On-flow LC–NMR spectra indicating the anomeric protons of the glycosidic unit and the characteristic olefinic proton of the major peaks (P1, P2, P3 and P4 going from lower to upper trace) from F1 under MeCN–$^{2}H_2O$ conditions. (Plotted with same expansion). (c) Comparison of the ^{1}H-NMR spectra of P4 from the active fraction F1 in MeOH–$^{2}H_2O$ (lower trace) and MeCN–$^{2}H_2O$ (upper trace), see text.

The molecular ion peak $[M+H]^+$ for both peaks 3 (P3) and 4 (P4) was seen at m/z 899 (Fig. 2). The diagnostic signals for P1 and P2 were seen at m/z 929, 779, 617, 455 and a base peak at 437 corresponding to $[M+H]^+$, $[M+H–arabinose]^+$, $[M+H–H_2O–arabinose–glucosyl]^+$, $[M+H–H_2O–arabinose–2$ x glucosyl$]^+$, and $[aglycone–H_2O]^+$, respectively. The signals for P3 were seen at m/z 899, 749, 587, 455 and a base peak at 437 corresponding to $[M+H]^+$, $[M+H–arabinose]^+$, $[M+H–arabinose–glucosyl]^+$, $[M+H–2$ x arabinosyl, glucose$]^+$ and $[aglycone–H_2O]^+$, respectively. The fragmentation pattern of P4 was similar to that of P3 with signals seen at m/z 899, 767, 605, 455 and 437. The analysis of the fragmentation pattern obtained in comparison with the literature data, implied the compounds to belong to the class of triterpenoidal saponins reported for the plant [8,9]. Also, it was clearly evident that we were dealing with two pairs of compounds having the same glycosidic chain. From literature, it seemed that the sugar units in P1 and P2 consisted of a terminal Glc and Ara units attached to an inner Glc unit containing the aglycone. Similarly, P3 and P4 seemed to have a terminal Glc and Ara units attached to an inner Ara unit containing the aglycone. The difference in the m/z values of the molecular ion peaks for the two pairs of compounds equivalent to 30 (corresponding to a CH_2O unit) further indicates that one of the structural differences in the two pairs of compounds is in the glycosidic chain of the molecule. Since the fragmentation pattern of the aglycone for all the four compounds was found to be similar, nothing much was inferred for the structural features of the aglycone.

Table 1
Selected characteristic ^{1}H-NMR chemical shifts of peaks P1, P2, P3 and P4 under MeCN–^2H_2O (assignments were aided by WETGCOSY and WETTOCSY experiments in the stop-flow mode)

	P1	P2		P3	P4
Glc 1′	4.45	4.45	Ara 1′	Obscured	Obscured
2′	3.47	3.47	2′	4.05	4.05
3′	3.72	3.72	3′	3.80	3.80
4′	3.48	3.48	4′	3.80	3.80
5′	3.29	3.29	5′	3.67, 3.75	3.67, 3.75
6′	3.79, 3.64	3.79, 3.64	–	–	–
Glc 1″	4.60	4.60	Glc 1″	4.52	4.52
2″	3.25	3.25	2″	3.40	3.40
3″	3.40	3.40	3″	3.34	3.34
4″	3.30	3.30	4″	3.28	3.28
5″	3.27	3.27	5″	3.36	3.36
6″	3.60, 3.82	3.60, 3.82	6″	3.78, 3.64	3.78, 3.64
Ara 1‴	5.40	5.40	Ara 1‴	5.25	5.25
2‴	4.10	4.10	2‴	4.06	4.06
3‴	3.95	3.95	3‴	3.91	3.91
4‴	4.00	4.00	4‴	3.97	3.97
5‴	3.70, 3.60	3.70, 3.60	5‴	3.68, 3.60	3.68, 3.60
18	1.08	1.00	–	1.08	1.00
19	0.83	0.78	–	0.83	0.78
21	1.13	0.97	–	1.13	0.97
22	1.46, 1.37	2.30	–	1.46, 1.37	2.30
23	4.60	4.07, 3.46	–	4.60	4.07, 3.46
24	5.10	5.20	–	5.10	5.20
26	1.60	1.66	–	1.60	1.66
27	1.70	1.58	–	1.70	1.58
28	1.00	0.92	–	1.00	0.92
29	0.80	0.72	–	0.80	0.72
30	4.03, 3.95	3.95, 3.89	–	4.03, 3.95	4.03, 3.95

The HPLC–NMR experiments were found to be sensitive enough to see the important signals both in the on-flow and stop-flow experiments. The on-flow ^{1}H-NMR spectrum of the active fraction indicated two regions of characteristic resonances for triterpenoidal glycosides, the methyl region ranging from the chemical shift values of δ 0.8 ppm to δ 1.9 ppm (Fig. 3a) and the sugar region ranging from the chemical shift values of δ 3.3 ppm and δ 5.6 ppm (Fig. 3b). Interestingly, comparison of the methyl region in acetonitrile–$^{2}H_2O$ run clearly indicated two pairs of compounds with similar aglycone (P1/P3 and P2/P4). Analysis of the downfield-region (δ 5.5→δ 4.0 ppm) furthermore confirmed two pairs of different aglycones. Analysis of the WETGCOSY spectra in the stop-flow mode for all four peaks

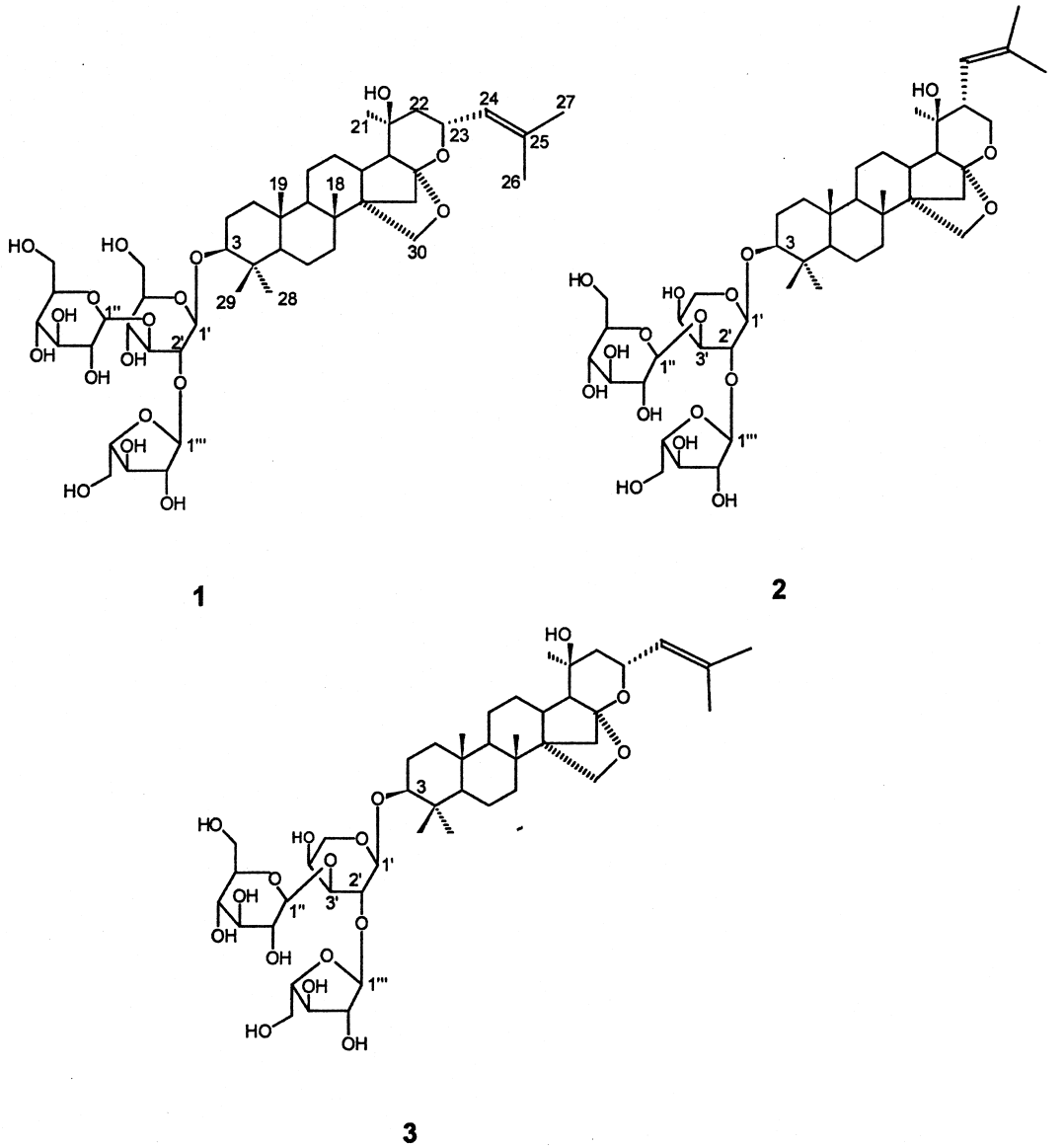

Fig. 4. Structures of the peaks P1, P3 and P4 corresponding to the chromatogram of F1. **1**=Bacoside A_3, **2**=3-β-[*O*-β-D-glucopyranosyl (1→3)-*O*-[α-L-arabinofuranosyl-(1→2)]-*O*-β-D-arabinopyranosyl) oxy] jujubogenin, **3**=bacopasaponin C.

supported the structural change to be a shift in the side chain consisting of C-24, C-25, C-26, C-27 from position 22 to 23. The WETGCOSY and the WET-TOCSY experiments supported the observations made from HPLC–MS that we were dealing with two pairs of compounds with similar sugar units. Apparently peaks P1/P2 as well as peaks P3/P4 bear the same sugar chain. Signals obscured by the solvent suppression could be observed by changing to methanol–$^{2}H_{2}O$ gradient, where solvent suppression takes place at different positions as seen in Fig. 3c. Careful analysis of the 1D and 2D spectra (see Table 1) suggested peaks 1, 3 and 4 to be 3-β-[*O*-β-D-glucopyranosyl-(1→3) *O*-[α-L-arabinofuranosyl-(1→2)]-*O*-β-D-glucopyranosyl) oxy] jujubogenin (bacoside A_3, **1**), 3-β-[*O*-β-D-glucopyranosyl (1→3)-*O*-[α-L-arabinofuranosyl-(1→2)]-*O*-β-D-arabinopyranosyl) oxy] jujubogenin (**2**) and 3-β-[*O*-β-D-glucopyranosyl (1→3)-*O*-[α-L-arabinofuranosyl-(1→2)]-*O*-β-D-arabinopyranosyl) oxy] pseudojujubogenin (bacopasaponin C, **3**), respectively, as shown in Fig. 4. The structures were confirmed by comparing the spectroscopical data with authentic samples.

On the other hand for P2, a careful analysis is necessary after isolation of the compound in order to clarify all the structural features. This is because the amount of the compound accessible under LC–NMR conditions is not enough to run the necessary HMBC, HSQC and NOESY spectra. It should be noted that one of the active saponins (P3, a known compound isolated from the bark of *Zizyphus Joazeiro*, Rhamnaceae) is reported for the first time from *B. monniera* [14].

4. Conclusions

The combined structural information from LC–NMR and LC–MS clearly show an insight into the existence of two pairs of compounds with the same sugar part and two pairs of compounds with similar aglycones. Interestingly the active compounds itself differ in both the aglycone and the nature of the sugar unit. However, isolation of one of the active compounds (P2) and detailed structural investigation including quantitative bioassay will be done. The results also showed the sensitivity of on-line coupled NMR experiments for identification of high-molecular-mass compounds. The hyphenated techniques including bioassay led to the rapid, efficient and straightforward investigation of compounds with close structural similarities without actually isolating them.

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References

[1] K. Albert, J. Chromatogr. A 703 (1995) 123.
[2] J.L. Wolfender, S. Rodriguez, K. Hostettmann, W. Hiller, Phytochem. Anal. 8 (1997) 97.
[3] I.D. Wilson, E.D. Morgan, R. Lafont, B. Wright, J. Chromatogr. A 799 (1998) 333.
[4] B. Vogler, I. Klaiber, G. Roos, C.U. Walter, W. Hiller, P. Sandor, W. Kraus, J. Nat. Prod. 61 (1998) 175.
[5] G. Roos, J.-H. Guse, I. Klaiber, B. Vogler, C.U. Walter, W. Kraus, Natural Product Analysis, Vieweg, Wiesbaden, 1998.
[6] R.N. Chopra, I.C. Chopra, K.L. Handa, L.D. Kapur, Chopra's Indegenenous Drugs of India, 2nd ed, Academic Publishers, Calcutta, New Delhi, 1982.
[7] D.K. Ganguly, C.L. Malhotra, Ind. J. Med. Res. 55 (1967) 473.
[8] S. Garai, S.B. Mahato, K. Ohtani, K. Yamasaki, Phytochemistry 42 (1996) 815.
[9] S. Rastogi, R. Pal, D.K. Kulshrestha, Phytochemistry 36 (1994) 133.
[10] P. Jain, D.K. Kulshrestha, Phytochemistry 33 (1993) 449.
[11] S. Garai, S.B. Mahato, K. Ohtani, K. Yamasaki, Phytochemistry 43 (1996) 447.
[12] K.G. Simkin, G.C. Coles, J. Chem. Tech. Biotechnol. 31 (1984) 419.
[13] S.H. Smallcombe, S.L. Patt, P.A. Keifer, J. Mag. Res. Ser. A 117 (1995) 295.
[14] R. Higuchi, S. Kubota, T. Komori, T. Kawasaki, V.B. Pandey, J.P. Singh, A.H. Shah, Phytochemistry 23 (1984) 2597.